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A20 inhibits LUBAC-mediated NF- B activation by binding linear polyubiquitin chains via its zinc finger 7

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(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 01 March 2012

Thank you for submitting your manuscript on A20 interference with linear ubiquitin chain signaling for consideration at The EMBO Journal. The study was sent to three reviewers, and we have at this stage already received the comments from two of them. Both of them are in pretty fair agreement that the study is potentially interesting and in principle suited for our journal, but that some further work - including essential strengthening of key data in endogenous, non-overexpression settings! - will be required before acceptance. In the interest of time, I have chosen to contact you with a preliminary decision on the basis of these first two reports and overlapping opinions. Therefore, I am inviting you to start revising the manuscript according to the referees' comments and suggestions already at this stage. As all their points appear well-taken and self-explanatory, I will not go through them in detail here, but still need to point out that it is EMBO Journal policy to allow a single round of revision only, and that it is thus essential that you completely answer the points raised if you wish the manuscript ultimately to be accepted. I should furthermore stress that this remains a preliminary decision and thus still subject to change should the last, missing report bring up serious additional concerns. Once the last report comes in, I will forward it to you and finalize my decision.

We generally allow three months as standard revision time. As a matter of policy, competing manuscripts published during this period will not negatively impact on our assessment of the conceptual advance presented by your study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed. Should you foresee a problem in meeting this three-month deadline, please let us know in advance and we may be able to grant an extension. When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be

available online to the community. For more details on our Transparent Editorial Process, please visit our website: http://www.nature.com/emboj/about/process.html

I hope you will find this early decision helpful, but should you need feedback on any further issue in this regard, please do not hesitate to get back to us.

Yours sincerely,

Editor
The EMBO Journal

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REFEREE REPORTS:

Referee #1

In this manuscript, Dr. Beyaert and colleagues investigate the role of the zinc finger protein A20 in the negative regulation of LUBAC-mediated NF-kB activation. The LUBAC E3 complex (HOIL-1, HOIP and Sharpin) conjugates linear polyubiquitin chains on the IKK subunit NEMO and has emerged as a critical activator of NF-kB in the TNFR and other pathways. However, little is known about the negative regulation of LUBAC and linear ubiquitination. The authors demonstrate TNF-inducible interactions between A20, NEMO and Sharpin. A20 inhibits NF-kB activation by overexpressed LUBAC in a zinc finger 7 (ZF7)-dependent manner. A20 ZF7 interacts with polyubiquitin chains (including linear chains), NEMO and HOIP/HOIL-1. Finally, A20 and A20 ZF7 disrupt the interactions between NEMO and Sharpin. This is a very interesting report that is the first to link A20 to the negative regulation of LUBAC and linear ubiquitination. However, some of the conclusions are based on overexpression studies in 293T cells and therefore additional experiments are needed to confirm several key points described below.

1) Fig. 1 (A and B) lacks the total IkBa control. The results should also be confirmed in cells other than 293T (i.e. macrophages).

2) In Figs. 2 and 3 the authors should show the expression levels of A20 and A20 mutants. These studies were performed in 293T cells which have high basal levels of A20 as the authors mentioned. It is also unclear if endogenous A20 is interacting with overexpressed forms of A20 and influencing NF-kB activation. Therefore, these experiments would be more conclusive if done in an A20-null (either knockout or siRNA knockdown) background. These figures could also benefit from supporting data showing the effects of A20 and ZF7 on IKK activation and/or the induction of NF-kB target genes.

3) In Fig. 6, the authors should include the A20 ZF4 mutant as a control.

4) Although the authors nicely demonstrate interactions between A20, NEMO and Sharpin and show that A20 ZF7 disrupts NEMO/Sharpin binding (Fig. 6), it is uncertain if A20 actually inhibits the linear ubiquitination of NEMO. Therefore, it will be important to determine if A20 removes linear Ub chains from NEMO in a ZF7-dependent manner. Ideally, the authors would examine the kinetics of TNF-induced NEMO linear ubiquitination using a linkage specific linear Ub antibody (see Tokunaga et al. 2009 Nat. Cell Biol. 11: 123-32) in control and A20-deficient cells. It can also be determined in the same experiment if NEMO/Sharpin interactions are enhanced or prolonged in the absence of A20.

Referee #2

In the manuscript 'A20 inhibits LUBAC-mediated NF-kB activation by binding linear polyubiquitin chains via its zinc finger 7', Verhelst and colleagues have shown a negative regulation mechanism of A20 in LUBAC-induced NF-kB activation that is independent from its catalytic activity. Verhelst et al. have for the first time shown that A20 and linear polyubiquitin chains interact via A20-ZF7. The

discovery of Verhelst has a high potential to attract the readers of EMBO Journal. However, there are some critical points that need to be clarified as described below.

Major points:
1. The major critical point, which still needs to be clarified is if the specific interaction between A20-ZF7 and linear polyubiquitin chains is required for the negative regulation by A20. From Figure 4A, it is clear that A20 ZF7 makes a complex with different linkage types of ubiquitin chains such as K48-linked, K63-linked and linear poly ubiquitin chains. In addition, the ZF7-mutant that abolishes the interaction with linear poly ubiquitin chains also loses the interaction with K48- or K63-linked polyubiquitin chains. These observations make the interpretation of the data throughout the manuscript not clear.
2. In figure 2 and 3, the inhibitory effect of A20-ZF7 on NF-kB activation is more efficient in LUBAC-induced NF-kB activation but not TNF-induced NF-kB activation. This suggests the importance of A20-ZF7 in the linear-ubiquitin dependent function rather than other ubiquitin chains. To partially answer the question as above (#1), the authors need to stress more about this in the text.
3. In figure 5A, the authors demonstrated that overexpressed NEMO and A20-FL or A20-ZF7 make a complex. Since the message of the manuscript is that the A20-ZF7 inhibits LUBAC-induced NF-kB signaling via interaction with linear polyubiquitin chains and NEMO is the major linear-ubiquitin substrate in this signaling, one would expect that A20-ZF7 interaction with NEMO happens when it is linearly ubiquitinated. The authors need to show if the interaction between ZF7 and NEMO increases either when the cells are stimulated by TNFa or when LUBAC is co-expressed.
4. In Figure 1B and figure 6, the interaction between A20 and sharpin or NEMO and sharpin is nicely shown. However, the authors claim that these interactions are between A20 and LUBAC or NEMO and LUBAC, HOIP and HOIL-1L need to be tested as well. It is very important especially to test HOIL-1L since there is a possibility that HOIP-HOIL complex, HOIP-sharpin complex or trimeric complex might have different effects on the A20-ZF7 function.
5. In figure 4B, input of linear polyubiquitin chains is missing. As K48- and K63-linked polyubiquitin chains are involved in A20-ZF7 interactions shown in Figure 4A, the direct interactions between ZF7 and chains using GST-ZF7 only wt and mutant and K48- or K63-linked poly ubiquitin chains need to be tested as well.
6. Throughout the manuscript, all the signaling experiments were done only in overexpressed system. To strengthen the data of the manuscript, the NF-kB signaling and the LUBAC-NEMO-A20 complex formation upon TNF stimulation in A20-wt or ZF7 mutant reconstituted cells in the background of A20-deficient cells (either A20 knockdown cells or A20 knock out cells) need to be tested.

Minor comment:
1. In page3, the last line: it is described that 'in contrast to HOIL-1 and HOIP, sharpin does not have enzymatic activity'. This is a confusing description because in the regulation of NF-kB signaling, only the catalytic action of HOIP is required but not HOIL-1L. This sentence needs to be rephrased.

Additional correspondence (editor) 07 March 2012

We have now received the attached third report on your manuscript. Although also this reviewer agrees on the potential importance of your findings and conclusions, s/he overall raises a number of more substantive criticisms regarding the presented experiments and the support they provide for the main interpretations. Most of these issues are well-taken and directly concern the data at hand, so I agree that it will essential to decisively address them before publication may be warranted.

In this light, I would nevertheless like to confirm my preliminary original decision to invite you to revise this study for The EMBO Journal. However, your revision will also have to address the points raised by referee 3, and eventual acceptance of the revised manuscript will depend on your satisfactory responses to the comments of all three referees. Should you have any questions in this regard, please do not hesitate to get back to me.

Yours sincerely,

Editor
The EMBO Journal

Referee 3

In this manuscript the authors pursue their study of the A20 protein, a ubiquitin ligase/DUB involved in negative regulation of the NF-κB activity. They show that the Zinc finger 7 (ZF7), which is part of the A20 domain exhibiting E3 ligase activity, is involved in binding to linear polyubiquitin chains. The authors show that following TNF stimulation, A20 is recruited to NEMO and LUBAC, leading to inhibition of NF-κB activation. They also show that this inhibition requires binding of A20 to linear polyUb chains through ZF7.

These results are potentially important, but the presented data are rather preliminary, and the conclusions are not adequately supported by substantial evidence. More specifically :

- figure 1 : these data do not demonstrate that NEMO, LUBAC and A20 are present in the same complex, they just show A20 recruitment to NEMO, which does not necessarily take place at the TNR receptor, as well as A20 recruitment to LUBAC, with the same caveat. Formal demonstration of the presence of a ternary complex requires two consecutive immunoprecipitations. Alternatively, immunoprecipitating the TNF receptor and demonstrating recruitment of NEMO, LUBAC and A20 would be more convincing that the presented data.
A second point raised by comparison of figure 5 with figure 1 : which subunit of LUBAC interacts with A20? Sharpin, HOIP, HOIL? All of them?

- figure 2 : the authors use LUBAC overexpression as an activator of NF-κB. However this is not a physiological mechanism of NF-κB activation, and it is unclear what physiological mechanism, if any, it is supposed to mimic. Therefore the results are difficult to interpret. In addition, comparison of figure 2A and 3A shows that A20 mutated in ZF7 is able to inhibit NF-κB activation by TNF but not by LUBAC, while ZF7-only is able to inhibit both. This is inconsistent and casts some doubt about the relevance of NF-κB activation by LUBAC. At least the authors should provide an explanation for these differences.

- figure 4A is a bit difficult to interpret, as immunoprecipitating A20 from transfected cells might pull down endogenous ubiquitin chains. A formal demonstration of the respective affinity of ZF7 or A20 for a specific type of chain would require affinity measurement, using recombinant A20 or ZF7. In panel B, controls using full length A20 and ZF4 should be shown.

- figure 5 raises an important issue : in the absence of stimulation (TNF for example), A20/ZF7 is not expected to interact with NEMO or HOIL/HOIP (A20 might exhibit some low basal affinity for NEMO or HOIL/HOIP, which incidently would be an important information, but this cannot be the case for ZF7). So why is the interaction detectable? Why do the authors use HOIP and HOIL while they have used sharpin in figure 1? They should demonstrate that overexpressed ZF7 interacts with endogenous NEMO and LUBAC, and that this occurs only following TNF stimulation, and demonstrate that this happens under conditions where either NEMO or LUBAC (HOIL, sharpin, ..?) is indeed polyubiquitinated.

- figure 6 : the postulated mechanism is not clear : does ZF7 interact with ubiquitinated NEMO, ubiquitinated LUBAC, both? Which one takes place first : it is difficult to envision that NEMO gets linearly ubiquitinated before encountering LUBAC, suggesting that A20 is recruited to LUBAC first. Is this the case? More

generally it is hard to imagine that ZF7 binding to linear chains would be sufficient to prevent binding of these chains to another protein. A peptide such as FP7 is expected to prevent binding of A20 to linear chains, but not binding of NEMO to LUBAC (whichever is ubiquitinated).

- Does ZF7 prevent binding of NEMO to ubiquitinated RIP1?

- in the Discussion, the authors propose a series of alternative interpretations of their results. In particular they write : "We cannot exclude the possibility that A20-ZF7 contributes to A20's NF-κB inhibitory activity by mechanisms that are independent of linear ubiquitination", indicating they don't seem to be convinced by their own experiments. They should perform additional experiments that allow a single mechanism to be proposed.

- minor point : bottom of page 3 : HOIL has no enzymatic activity either.

1st Revision - authors' response 13 July 2012

Reply to the referees:

We gratefully appreciate the three reviewers' comments on our manuscript, which allowed us to significantly improve the manuscript. We have addressed all the comments in a point-by-point response below. We also provide the results of several additional experiments in our revision.

Referee 1:

In this manuscript, Dr. Beyaert and colleagues investigate the role of the zinc finger protein A20 in the negative regulation of LUBAC-mediated NF-kB activation. The LUBAC E3 complex (HOIL-1, HOIP and Sharpin) conjugates linear polyubiquitin chains on the IKK subunit NEMO and has emerged as a critical activator of NF-kB in the TNFR and other pathways. However, little is known about the negative regulation of LUBAC and linear ubiquitination. The authors demonstrate TNF-inducible interactions between A20, NEMO and Sharpin. A20 inhibits NF-kB activation by overexpressed LUBAC in a zinc finger 7 (ZF7)-dependent manner. A20 ZF7 interacts with polyubiquitin chains (including linear chains), NEMO and HOIP/HOIL-1. Finally, A20 and A20 ZF7 disrupt the interactions between NEMO and Sharpin. This is a very interesting report that is the first to link A20 to the negative regulation of LUBAC and linear ubiquitination. However, some of the conclusions are based on overexpression studies in 293T cells and therefore additional experiments are needed to confirm several key points described below.

> *We thank this reviewer for evaluating our study as very interesting and novel. Our reply to the specific comments is given below.*

1) Fig. 1 (A and B) lacks the total IkBa control.

> *IkBa is now shown in the revised figure 1. For figure 1A, we did not have the original blots anymore and we therefore performed the whole experiment again with similar results. The original figure 1A has been replaced with the results of this new experiment.*

The results should also be confirmed in cells other than 293T (i.e. macrophages).

> *We confirmed the initial results obtained in HEK293T cells also in Jurkat cells, which are now shown in figure 1C and 1D.*
> *In addition, we included another novel experiment showing the recruitment of A20, together with NEMO and LUBAC, in the TNF-R complex of TNF stimulated Jurkat cells (shown in a novel figure 2).*

2) In Figs. 2 and 3 the authors should show the expression levels of A20 and A20 mutants.

> *We now show the expression levels for A20 and A20 mutants in the NF-κB reporter gene assays (figures 3 en 4 in the revised manuscript) in a supplementary figure 1, showing equal expression levels for WT A20 and the corresponding mutants.*

These studies were performed in 293T cells which have high basal levels of A20 as the authors mentioned. It is also unclear if endogenous A20 is interacting with overexpressed forms of A20 and influencing NF-kB activation. Therefore, these experiments would be more conclusive if done in an A20-null (either knockout or siRNA knockdown) background. These figures could also benefit from supporting data showing the effects of A20 and ZF7 on IKK activation and/or the induction of NF-kB target genes.

> *To analyze if endogenous A20 is interacting with $ZF7_{only}$, we investigated if endogenous A20 could be coimmunoprecipitated with $ZF7_{only}$ in transfected HEK293T cells. Figure 7B and 7C show that, although all LUBAC components and NEMO nicely interact with the A20 mutant, we could not detect any interaction with endogenous A20, making a role for endogenous A20 in the NF-κB inhibitory effect of $ZF7_{only}$ very unlikely.*
> *To further exclude a role for endogenous A20, and in response to the reviewer's request to also analyze the effect of A20 and the $ZF7_{only}$ mutant in an A20-null background and to show their effect on the induction of an NF-κB target gene, we reconstituted A20 knockout MEF cells with either wild type A20 or $ZF7_{only}$ and analyzed the induction of iNOS expression in response to TNF. As can be seen from the novel figure 5, $ZF7_{only}$ and WT A20 significantly inhibited TNF-induced iNOS expression in an A20-null background. These results further exclude a role for endogenous A20 in the observed effect of ZF_{only} and validate the original NF-κB reporter gene results for a physiological NF-κB target gene.*

3) In Fig. 6, the authors should include the A20 ZF4 mutant as a control.

> *The experiment shown in the original figure 6 (figure 8 in the revised manuscript) showed that expression of wild type A20, but not A20 in which ZF7 is mutated (A20-ZF7*), prevents the TNF-induced binding of NEMO to LUBAC. In response to the reviewer's request, we repeated this experiment and included A20 with a mutation in ZF4 (A20-ZF4*) as a control. As shown before (figure 8), A20-WT again completely prevented the TNF-induced interaction between NEMO and Sharpin. However, whereas mutation of ZF7 abolished the effect of A20, mutation of ZF4 did not. The results of these experiments are shown in the revised manuscript as supplementary figure 3.*

4) Although the authors nicely demonstrate interactions between A20, NEMO and Sharpin and show that A20 ZF7 disrupts NEMO/Sharpin binding (Fig. 6), it is uncertain if A20 actually inhibits the linear ubiquitination of NEMO. Therefore, it will be important to determine if A20 removes linear Ub chains from NEMO in a ZF7-dependent manner.

> *It is unlikely that A20 prevents NEMO/Sharpin binding by removing linear ubiquitin chains from NEMO or LUBAC components in a ZF7-dependent manner, as recombinant A20 was previously shown to specifically deubiquitinate K11- and K48-linked chains, but not linear polyubiquitin (Bosanac et al., 2010; Komander and Barford, 2008). Similarly, we were unable to detect any DUB activity on linear polyubiquitin with A20 immunoprecipitated from transfected cells (shows as supplementary figure 4 in the revised manuscript). Moreover, the fact that NEMO/LUBAC complex formation can already be disrupted by A20-$ZF7_{only}$ which lacks any DUB activity excludes a role for A20-mediated removal of linear ubiquitin from NEMO or LUBAC.*

Ideally, the authors would examine the kinetics of TNF-induced NEMO linear ubiquitination using a linkage specific linear Ub antibody (see Tokunaga et al. 2009 Nat. Cell Biol. 11: 123-32) in control and A20-deficient cells. It can also be determined in the same experiment if NEMO/Sharpin interactions are enhanced or prolonged in the absence of A20.

We were unable to study the linear ubiquitination of NEMO as suggested by the reviewer because we did not have access to good linear ubiquitin linkage-specific antibodies. However, for reasons mentioned above, an effect of A20 on the linear ubiquitination of NEMO itself is unlikely.
In response to the suggestion of the reviewer to also determine if NEMO/Sharpin interaction is enhanced or prolonged in the absence of A20, we tried to knockdown A20 using RNA interference. However, we were unable to obtain a sufficient knockdown using several strategies (transient transfection of siRNA or esiRNA, as well as stable transfection of shRNA) and A20 target sequences. Over the years, we have experienced that knockdown of A20 is not well tolerated by the cells, which may reflect the known anti-apoptotic activities of A20 (Opipari et al., J Biol Chem. 1992 Jun 25;267(18):12424-7).

Referee 2:

In the manuscript 'A20 inhibits LUBAC-mediated NF-kB activation by binding linear polyubiquitin chains via its zinc finger 7', Verhelst and colleagues have shown a negative regulation mechanism of A20 in LUBAC-induced NF-kB activation that is independent from its catalytic activity. Verhelst et al. have for the first time shown that A20 and linear polyubiquitin chains interact via A20-ZF7. The discovery of Verhelst has a high potential to attract the readers of EMBO Journal. However, there are some critical points that need to be clarified as described below.

We thank this reviewer for the positive comments and provide our reply to the specific comments below.

Major points:

1. The major critical point, which still needs to be clarified, is if the specific interaction between A20-ZF7 and linear polyubiquitin chains is required for the negative regulation by A20. From Figure 4A, it is clear that A20 ZF7 makes a complex with different linkage types of ubiquitin chains such as K48-linked, K63-linked and linear poly ubiquitin chains. In addition, the ZF7-mutant that abolishes the interaction with linear poly ubiquitin chains also loses the interaction with K48- or K63-linked polyubiquitin chains. These observations make the interpretation of the data throughout the manuscript not clear.

We agree with the reviewer that the data shown in Fig. 4A of the original manuscript, showing binding of ZF7 to all three types of ubiquitin chains, made the interpretation of the data sometimes difficult. It should be mentioned that these data were obtained with ubiquitin binding assays in which we used GFP-A20 and GFP-ZF7$_{only}$ mutants that were immunoprecipitated from transfected cells. Under these conditions, we can not completely exclude the presence of other A20-binding proteins that might be responsible for the observed lack of ubiquitin chain specificity. We therefore repeated the experiment using recombinant GST-fusion proteins that were expressed and purified from bacteria. Using these tools, we now show that ZF7 almost exclusively binds linear polyubiquitin (in the revised manuscript we replaced the old figure 4A with a new figure 6A showing these novel data). Disruption of the zinc finger structure by mutation of C775A-C779A completely abolished the interaction of ZF7 with linear polyubiquitin. We also assessed the binding of ZF7 to linear ubiquitin chains in competition with K48- or K63-linked polyubiquitin chains of the same length as recently described (Kensche et al., 2012). Purified ZF7 was incubated with equal concentrations of different types of polyubiquitin in the same tube and binding of ZF7 was monitored by washing off unbound chains and western blot analysis. Since ubiquitin chains of the same length but with different linkage display different mobility on SDS-polyacrylamide gel electrophoresis, it is possible to distinguish the different chain types by using the same anti-ubiquitin antibody. Incubation of ZF7 with different ubiquitin chain types led to a preferential binding of linear polyubiquitin in competition with either K48- or K63-polyubiquitin (Fig. 6B of the revised manuscript). These data identify ZF7 as a specific linear ubiquitin-binding domain (LUBID). Together with our other observations, including additional novel data showing that ZF7 interacts with NEMO in a TNF and LUBAC-inducible manner (Fig. 7) and prevents LUBAC-induced NF-KB activation, these

data further support an important role for ZF7 binding to linear ubiquitin in the negative regulation of NF-κB by A20.

2. In figure 2 and 3, the inhibitory effect of A20-ZF7 on NF-kB activation is more efficient in LUBAC-induced NF-kB activation but not TNF-induced NF-kB activation. This suggests the importance of A20-ZF7 in the linear-ubiquitin dependent function rather than other ubiquitin chains. To partially answer the question as above (#1), the authors need to stress more about this in the text.

We thank the reviewer for making this important point and we have stressed this more in the discussion of the revised manuscript as mentioned below:
"The ability of ZF4 and ZF7 to bind different types of polyubiquitin chains may also reflect our observation that complete disruption of A20's inhibitory activity on TNF-induced NF-κB activation requires mutation of both ZF4 and ZF7, whereas mutation of ZF7 is sufficient to disrupt A20's inhibitory activity on NF-κB activation induced by LUBAC overexpression. Specific binding of linear chains by ZF7 may allow A20 to affect linear ubiquitin-mediated NEMO/LUBAC binding, whereas binding of ZF4 to K63-polyubiquitin may allow the recognition by A20 of upstream K63-ubiquitinated signaling molecules such as RIP1 and TRAF2."

3. In figure 5A, the authors demonstrated that overexpressed NEMO and A20-FL or A20-ZF7 make a complex. Since the message of the manuscript is that the A20-ZF7 inhibits LUBAC-induced NF-kB signaling via interaction with linear polyubiquitin chains and NEMO is the major linear-ubiquitin substrate in this signaling, one would expect that A20-ZF7 interaction with NEMO happens when it is linearly ubiquitinated. The authors need to show if the interaction between ZF7 and NEMO increases either when the cells are stimulated by TNF or when LUBAC is co-expressed.

We thank the reviewer for this suggestion. We have performed the requested experiments for which the results are shown in figure 7B and 7C. Figure 7B shows that TNF indeed increases the interaction between ZF7 and endogenous NEMO. In addition, figure 7C shows that the interaction between ZF7 and NEMO is also stimulated by LUBAC overexpression. These results strongly indicate that ZF7 interacts with NEMO when it is linearly ubiquitinated.

4. In Figure 1B and figure 6, the interaction between A20 and sharpin or NEMO and sharpin is nicely shown. However, the authors claim that these interactions are between A20 and LUBAC or NEMO and LUBAC, HOIP and HOIL-1L need to be tested as well. It is very important especially to test HOIL-1L since there is a possibility that HOIP-HOIL complex, HOIP-sharpin complex or trimeric complex might have different effects on the A20-ZF7 function.

In the revised manuscript we have extended these experiments to HOIP and HOIL-1, in addition to Sharpin (shown in Fig. 1, Fig. 2, Fig. 7A and 7C, and suppl. Fig. 2), showing that A20 and NEMO interact with all three LUBAC components.

5. In figure 4B, input of linear polyubiquitin chains is missing.
As K48- and K63-linked polyubiquitin chains are involved in A20-ZF7 interactions shown in Figure 4A, the direct interactions between ZF7 and chains using GST-ZF7 only wt and mutant and K48- or K63-linked poly ubiquitin chains need to be tested as well.

As mentioned above (reply to first comment), we replaced figure 4B with a new figure 6A in which we tested the binding of recombinant GST-ZF7 wild type and mutant fusion proteins to K48-, K63- and linear linked ubiquitin chains. For each chain type, also input is now shown. Moreover, additional data shown in figure 6B (competition experiments) further strengthen our conclusions.

6. Throughout the manuscript, all the signaling experiments were done only in overexpressed system. To strengthen the data of the manuscript, the NF-kB signaling and the LUBAC-NEMO-A20 complex formation upon TNF stimulation in A20-wt or ZF7 mutant reconstituted cells in the background of A20-deficient cells (either A20 knockdown cells or A20 knock out cells) need to be tested.

In the revised manuscript we now show the results of an experiment in which we reconstituted A20 knockout MEF cells with either wild type A20 or $ZF7_{only}$ and analyzed the induction of iNOS (an NF-κB target gene) expression in response to TNF. As can be seen from the novel figure 5, $ZF7_{only}$ and WT A20 significantly inhibited TNF-induced iNOS expression in an A20-null background.
We were unable to also study LUBAC-NEMO-A20 complex formation in these cells (mouse embryonic fibroblasts) because the lack of antibodies that recognize mouse LUBAC components with sufficient specificity. As an alternative, we tried to knockdown A20 in human cells, but in this case we were unable to obtain a sufficient knockdown using several strategies (transient transfection of siRNA or esiRNA, as well as stable transfection of shRNA) and A20 target sequences. Over the years, we have experienced that knockdown of A20 is not well tolerated by the cells, which may reflect the known anti-apoptotic activities of A20 (Opipari et al., J Biol Chem. 1992 Jun 25;267(18):12424-7).

Minor comment:
1. In page3, the last line: it is described that 'in contrast to HOIL-1 and HOIP, sharpin does not have enzymatic activity'. This is a confusing description because in the regulation of NF-kB signaling, only the catalytic action of HOIP is required but not HOIL-1L. This sentence needs to be rephrased.

Indeed, this was wrongly mentioned. We have corrected this in the revised manuscript (first sentence of page 4).

Referee 3:

In this manuscript the authors pursue their study of the A20 protein, a ubiquitin ligase/DUB involved in negative regulation of the NF-kB activity. They show that the Zinc finger 7 (ZF7), which is part of the A20 domain exhibiting E3 ligase activity, is involved in binding to linear polyubiquitin chains. The authors show that following TNF stimulation, A20 is recruited to NEMO and LUBAC, leading to inhibition of NF-kB activation. They also show that this inhibition requires binding of A20 to linear polyUb chains through ZF7. These results are potentially important, but the presented data are rather preliminary, and the conclusions are not adequately supported by substantial evidence. More specifically:

- figure 1 : these data do not demonstrate that NEMO, LUBAC and A20 are present in the same complex, they just show A20 recruitment to NEMO, which does not necessarily take place at the TNR receptor, as well as A20 recruitment to LUBAC, with the same caveat. Formal demonstration of the presence of a ternary complex requires two consecutive immunoprecipitations. Alternatively, immunoprecipitating the TNF receptor and demonstrating recruitment of NEMO, LUBAC and A20 would be more convincing than the presented data.

We thank the reviewer for this suggestion. In response, we performed an additional experiment in which we immunoprecipitated the TNF receptor complex from Flag-TNF-stimulated Jurkat cells and analyzed the recruitment of A20, NEMO and LUBAC components. Anti-Flag immunoprecipitation showed the recruitment of A20 along with NEMO and all three LUBAC components to the TNF-R after 5 min TNF stimulation (Fig. 2 of the revised manuscript). Interestingly, A20 levels in the TNF-R complex still further increased after 15 min TNF stimulation, whereas binding of NEMO and LUBAC already declined, consistent with the proposed competitive binding of A20 with linearly ubiquitinated NEMO and LUBAC.

A second point raised by comparison of figure 5 with figure 1 : which subunit of LUBAC interacts with A20? Sharpin, HOIP, HOIL? All of them?

In the revised manuscript we have analyzed the interaction of A20 with the different LUBAC components. Multiple experiments (shown in Fig. 1, Fig. 2, Fig. 7A and 7C), show that A20 interacts with all three LUBAC components.

- figure 2: the authors use LUBAC overexpression as an activator of NF-kB. However this is not a physiological mechanism of NF-kB activation, and it is unclear what physiological mechanism, if

any, it is supposed to mimic. Therefore the results are difficult to interpret. In addition, comparison of figure 2A and 3A shows that A20 mutated in ZF7 is able to inhibit NF-kB activation by TNF but not by LUBAC, while ZF7-only is able to inhibit both. This is inconsistent and casts some doubt about the relevance of NF-kB activation by LUBAC. At least the authors should provide an explanation for these differences.

NF-κB activation by overexpression of LUBAC (or other NF-κB signaling molecules) has been used in several other published studies (Kensche et al., JBC, 2012; Gerlach et al., Nature 471, 2011; Tokunaga et al., Nature Cell Biology 11, 2009), but we agree that overexpression of LUBAC is not a physiological mechanism of NF-kB activation. However, it can be used to bypass (at least partially) the TNF-induced activation of some upstream signaling molecules by other types of modification, including their known modification and regulation by K48- and K63-linked ubiquitination), and to obtain some information on the specific signaling steps that are targeted by ZF4 and ZF7 of A20.
Our observation that complete disruption of A20's inhibitory activity on TNF-induced NF-κB activation requires mutation of both ZF4 and ZF7, whereas mutation of ZF7 is sufficient to disrupt A20's inhibitory activity on NF-κB activation induced by LUBAC overexpression is consistent with the ability of ZF4 and ZF7 to bind different types of polyubiquitin chains. Specific binding of linear ubiquitin chains by ZF7 may allow A20 to affect linear ubiquitin-mediated NEMO/LUBAC binding, whereas binding of ZF4 to K63-polyubiquitin may allow the recognition by A20 of upstream K63-ubiquitinated signaling molecules such as RIP1 and TRAF2. These data therefore suggest the importance of A20-ZF7 in the linear-ubiquitin dependent function of A20. This was also put forward by reviewer 2 (comment 2), who asked us to stress this more in the text. In response, we emphasized this more in the discussion of the revised manuscript.

- figure 4A is a bit difficult to interpret, as immunoprecipitating A20 from transfected cells might pull down endogenous ubiquitin chains. A formal demonstration of the respective affinity of ZF7 or A20 for a specific type of chain would require affinity measurement, using recombinant A20 or ZF7. In panel B, controls using full length A20 and ZF4 should be shown.

These remarks are related to comments 1 and 5 of reviewer 2.
We agree with the reviewer that immunoprecipitating A20 from transfected cells might pull down endogenous ubiquitin chains and complicate the interpretation of the data. We therefore repeated the experiment using recombinant GST-fusion proteins that were expressed and purified from bacteria. Using these tools, we now show that ZF7 almost exclusively binds linear polyubiquitin (in the revised manuscript we replaced the old figure 4A with a new figure 6A showing these novel data). Disruption of the zinc finger structure by mutation of C775A-C779A completely abolished the interaction of ZF7 with linear polyubiquitin. We also assessed the binding of ZF7 to linear ubiquitin chains in competition with K48- or K63-linked polyubiquitin chains of the same length as recently described (Kensche et al., 2012). Purified ZF7 was incubated with equal concentrations of different types of polyubiquitin in the same tube and binding of ZF7 was monitored by washing off unbound chains and western blot analysis. Since ubiquitin chains of the same length but with different linkage display different mobility on SDS-polyacrylamide gel electrophoresis, it is possible to distinguish the different chain types by using the same anti-ubiquitin antibody. Incubation of ZF7 with different ubiquitin chain types led to a preferential binding of linear polyubiquitin in competition with either K48- or K63-polyubiquitin (Fig. 6B of the revised manuscript). These data identify ZF7 as a specific linear ubiquitin-binding domain (LUBID). Together with our other observations, including additional novel data showing that ZF7 interacts with NEMO in a TNF and LUBAC-inducible manner (Fig. 7) and prevents LUBAC-induced NF-KB activation, these data further support an important role for ZF7 binding to linear ubiquitin in the negative regulation of NF-κB by A20.
In vitro incubation of different types of polyubiquitin with recombinant Flag-A20 followed by western blotting and detection with anti-ubiquitin, revealed the potential of full length A20 to bind linear as well as K48- and K63-linked polyubiquitin chains (Fig. 6A). Most likely this reflects a role for other ZFs including A20-ZF4, which was previously shown to have higher affinity for K63-linked tri-ubiquitin than for K48-linked or linear tri-ubiquitin (Bosanac et al., 2010).

- figure 5 raises an important issue : in the absence of stimulation (TNF for example), A20/ZF7 is not expected to interact with NEMO or HOIL/HOIP (A20 might exhibit some low basal affinity for NEMO or HOIL/HOIP, which incidently would be an important information, but this cannot be the case for ZF7). So why is the interaction detectable?

> *This question was also partially raised by referee 2 in comment 3.*
> *We therefore performed two novel experiments showing that TNF treatment increases the interaction between ZF7 and endogenous NEMO (Fig. 7B). In addition, figure 7C shows that the interaction between ZF7 and NEMO is also stimulated by LUBAC overexpression. These results strongly indicate that ZF7 interacts with NEMO when it is linearly ubiquitinated.*
> *It should be noted that one can already observe a weak constitutive interaction between ZF7 and NEMO in both experiments, which was however strongly increased upon stimulation. We speculate that the constitutive interaction may reflect some basal linear ubiquitination induced by cellular stress due to the transfection procedure.*

Why do the authors use HOIP and HOIL while they have used sharpin in figure 1?
They should demonstrate that overexpressed ZF7 interacts with endogenous NEMO and LUBAC, and that this occurs only following TNF stimulation, and demonstrate that this happens under conditions where either NEMO or LUBAC (HOIL, sharpin, ..?) is indeed polyubiquitinated.

> *At the time we performed these experiments we did not yet have a plasmid encoding Sharpin. Now we also included Sharpin in the revised figure 7C and show that all three LUBAC components can bind to ZF7.*
> *In figure 7A and 7B of the revised manuscript we now show that overexpressed ZF7 can interact with endogenous HOIL-1, HOIP, Sharpin and NEMO following TNF stimulation. We also investigated the ubiquitination of NEMO and Sharpin under these conditions, but the ubiquitination signal that we detected with anti-ubiquitin was too weak to allow any reliable conclusions. Instead, we performed an additional experiment in which we show that binding of ZF7 to NEMO is also strongly increased upon LUBAC overexpression (Fig. 7C), which is supposed to induce the linear ubiquitination of NEMO.*

- figure 6 : the postulated mechanism is not clear: does ZF7 interact with ubiquitinated NEMO, ubiquitinated LUBAC, both? Which one takes place first : it is difficult to envision that NEMO gets linearly ubiquitinated before encountering LUBAC, suggesting that A20 is recruited to LUBAC first. Is this the case?

> *The question related to the timing of the different ubiquitination events and the recruitment of different signaling components in the TNF-R complex is highly interesting but difficult to address. As also shown in our experiments, the recruitment of A20 and LUBAC in the TNF-R complex occurs very rapidly (within 5 min) after TNF stimulation, making it very difficult to say which one comes first. Also the stoichiometry of the TNF-R signaling complex is still largely unclear and it is possible that other signaling components become linearly ubiquitinated in addition to NEMO, LUBAC, and RIP1. Moreover, many other signaling molecules in the TNF-R complex are known to be modified by other types of ubiquitination, some of which will also contribute to the recruitment of A20 (e.g. via ZF4 which recognizes K63-linked polyubiquitin), NEMO and LUBAC. The investigation of this will need significant efforts and time, and is in our opinion beyond the scope of the current study. We hope that this referee can appreciate this.*

More generally it is hard to imagine that ZF7 binding to linear chains would be sufficient to prevent binding of these chains to another protein. A peptide such as ZF7 is expected to prevent binding of A20 to linear chains, but not binding of NEMO to LUBAC (whichever is ubiquitinated).

> *We provide multiple evidence that binding of ZF7 to linear ubiquitin chains is associated with the inhibition of NEMO-LUBAC interaction and TNF/LUBAC-induced NF-κB activation, supporting a model in which binding of A20 to linear ubiquitinated NEMO or LUBAC prevents their mutual binding. As mentioned in the discussion we do not exclude*

that also other mechanisms (such as inhibition of E2/LUBAC binding) contribute to the inhibitory effect of ZF7. However, this will be the topic of future research projects in our lab.

- Does ZF7 prevent binding of NEMO to ubiquitinated RIP1?

Since RIP1 is known to be also modified by linear ubiquitin chains, it could indeed be hypothesized that A20 will also prevent the interaction between NEMO and RIP1. We initially tried to study this but failed to reproducibly detect an interaction between NEMO and RIP1 upon TNF stimulation. We therefore decided to focus on NEMO and LUBAC in our experiments.

- in the Discussion, the authors propose a series of alternative interpretations of their results. In particular they write : "We cannot exclude the possibility that A20-ZF7 contributes to A20's NF-kB inhibitory activity by mechanisms that are independent of linear ubiquitination", indicating they don't seem to be convinced by their own experiments. They should perform additional experiments that allow a single mechanism to be proposed.

Although our findings support a model in which A20 prevents the binding of NEMO to LUBAC via competitive binding to linear polyubiquitin chains, we indeed do not exclude that also other mechanisms, in addition to linear ubiquitin binding, may be involved in certain conditions or certain cell types. We have now explained this a bit more in detail in the revised discussion (page 17). We believe that the discussion section is the right place to bring this to the attention of the readers as it may offer some interesting perspectives for future work. However, this does not mean at all they we are not convinced by our own experiments.

- minor point : bottom of page 3 : HOIL has no enzymatic activity either.

Indeed, this was wrongly mentioned. We have corrected this in the revised manuscript (first sentence of page 4).

2nd Editorial Decision 24 July 2012

Thank you for submitting your revised manuscript for our consideration. Two of the original reviewers have now reviewed it once more (see comments below), and I am pleased to inform you that they have no more principle objections towards publication in The EMBO Journal. Before we shall be able to proceed with formal acceptance of the paper, there are however a number of important editorial issues to be still taken care of:

- please include a brief Conflict of Interest statement in the text, next to the Author Contribution section

- please combine all supplementary information (text & figures) into a single PDF of sufficient quality, to facilitate download by our readers

- data in several of the blot panels remain difficult to assess because of image quality and/or contrast/brightness settings that lead to loss of background signals. Please revise the respective figure panels by including less adjusted images in Figs 1B (A20 panel), 1C (HOIL-1 panels, IkBa panel), 1D (IkBa panel), 6B (Ub panels), 7A (P-IkBa panel), 7B (GFP panels, and see also misplaced division line in NEMO panels), 7C (FLAG, GFP panels)

- the blot data in Figure 5 need an alternative presentation that allows to assess how they correspond to each other on a single gel/blot. At the very least, the source data file showing the origin of each of

these single bands needs to be included. The actin blots are again devoid of background due to excessive brightness

- please also revise the images in Supplementary Information - here the majority of panels show hyper-adjusted contrast/brightness setting, loss of background signals, and sometimes (e.g. S1B) even very low resolution. Also division lines and panel borders are often misplaced, e.g. in S1C, S3 (NEMO TL row). Therefore please carefully revise all supplementary figures to improve data representation and overall presentation.

- finally, in order to complement the required image improvement efforts, please also provide source data files for the various electrophoretic gels and blots in this case. We would ask for a single PDF/JPG/GIF file per figure comprising the original, uncropped and unprocessed scans of all gel/blot panels used in the respective figures. These should be labelled with the appropriate figure/panel number, and should have molecular weight markers; further annotation would clearly be useful but is not essential. A ZIP archive containing these individual files can be uploaded upon resubmission (selecting "Figure Source Data" as object type) and would be published online with the article as a supplementary "Source Data" file.

I am therefore returning the manuscript to you for one final round of minor revision, hoping you will be able to upload and re-submit the final corrected version as soon as possible. Should you have any questions in this regard, please do not hesitate to contact me directly.

Yours sincerely,
Editor
The EMBO Journal

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REFEREE REPORTS:

Referee #1

The authors have considerably improved the manuscript. In my opinion, it is now suitable for publication.

Referee #2

Verhelst and her colleagues demonstrated in their manuscript 'A20 inhibits LUBAC-mediated NF-kB activation by binding linear polyubiquitin chains via its zinc finger 7' that A20 ZF7 is a critical domain for the inhibitory effects on NF-kB signaling. In the revised manuscript, they responded to all the points from the referee 2 and the manuscript has been significantly improved. Especially the specificity of ZF7 to linear ubiquitin chains has been clarified using in vitro system and the authors provided further evidences of the functions of ZF7 by using MEFs and Jurkat cells. The revised manuscript has a clear message with high quality of data therefore the referee considers it suitable to be published in the EMBO Journal.

2nd Revision - authors' response 02 August 2012

Thank you for the rapid reviewing of our revised manuscript. We are happy to read that the referees have no more principle objections and that you are willing to accept our manuscript for publication after some minor revisions.

We have addressed all editorial issues as mentioned in your e-mail of July 24th.

A brief conflict of interest statement has been added after the Author Contributions section.

Revised figures (including also those for supplementary information) are now provided in which image quality has been improved and contrast/brightness settings have been changed so that background signals are visible.

Supplementary information (text and figures) is now provided in a single pdf file.

Regarding the remark made on the presentation of expression levels in figure 5, we prefer to keep it in the way it was orignally presented but provide some more information in the legend. We added the following to the legend: "Expression levels of transfected GFP-A20 fusion proteins were verified via western blotting with anti-GFP. All samples are from the same experiment; ZF7only and ZF7*only samples (non-stimulated and stimulated, each in triplicate) were loaded on the same gel, but WT triplicates were loaded on a separate gel. Gels/blots were processed in parallel and loading controls (anti-actin) are from the same gel/blot and from the same lane as the corresponding GFP-A20 fusion protein. A representative signal is shown at the bottom of the graph." We have also provided the source data file showing the origin of the single bands.

3rd Editorial Decision 03 August 2012

Thank you for sending in your re-revised manuscript as well as all the complete set of figure source data. I have now briefly assessed all this material as well as the final changes to the manuscript, and I am happy to inform you that we are now ready to accept your article for publication in The EMBO Journal.

You shall shortly receive a formal letter of acceptance, describing details on the further proceedings.

Thank you once again for this contribution to The EMBO Journal and congratulations on a successful publication. Please consider us again in the future for your most exciting work.

Yours sincerely,

Editor

The EMBO Journal

